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Modification of Resistance of Mice to *Naegleria fowleri* Infections

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Naegleria fowleri, which produces a fatal meningoencephalitis in humans, is also able to produce a progressive and fatal disease in mice. The course of the disease in DUB/ICR mice is dependent upon the infecting dose of organisms, whether administered intraperitoneally (i.p.) or intravenously (i.v.). All of the mice receiving 10^7 trophozoites/mouse i.v. or 4.85×10^7 trophozoites/mouse i.p. were killed within 10 days. *Escherichia coli* O26:B6 lipopolysaccharide, administered at a dose of 1 mg/kg 24 h prior to *N. fowleri*, afforded some protection for several days after challenge, but by day 8 there was no difference in survival of untreated and endotoxin-treated mice. No significant protection was afforded by a complex of lipid A with concanavalin A (ConA) or bovine serum albumin (BSA) or by dimethylmyristamide-BSA, dimethylmyristamide, BSA, β-hydroxymyristic acid-ConA, β-hydroxymyristic acid, ConA, myristic acid-BSA, or myristic acid. Mice surviving primary i.v. or i.p. challenge doses of *N. fowleri*, 5×10^6 and 10^7 trophozoites/mouse, respectively, were highly resistant to rechallenge with an i.v. dose of organisms (5×10^6 *Naegleria*/mouse) that produced uniformly fatal disease in untreated control mice.

Primary amoebic meningoencephalitis is a worldwide, albeit rather rare, fatal disease of humans. Outbreaks of primary amoebic meningoencephalitits have been reported in Australia, Czechoslovakia, and the United States of America. The etiological agent of primary amoebic meningoencephalitis in humans is *Naegleria fowleri* (7).

The mouse is uniquely appropriate as an experimental model for studying primary amoebic meningoencephalitis. The basic features of the disease in humans and mice are the same with respect to incubation period, portal of entry, residence of amoebae in the olfactory mucosa with invasion and migration through submucosal structures and into nerve plexuses, passage of amoebae through pores of the cribriform plate and into subarachnoid space, and subsequent invasion of olfactory bulbs and lobes with spread to more distant areas of the brain (10).

Treatment of patients with naeglerial infections has generally been unsuccessful (1). Earlier chemotherapy included antibacterial agents and antiprotozoal drugs. Amphotericin B is the only drug that has shown promise for treating a naeglerial infection (11). Accordingly, immunological approaches to the prevention and treatment of primary amoebic meningoencephalitis are warranted.

Many workers have established that bacterial endotoxins enhance or inhibit the pathogenicity of an infection, depending on the infecting microorganism, dose, and route of injection of endotoxin and the interval between administration of toxin and initiation of infection. Alteration by endotoxin of host resistance to infectious diseases caused by protozoa (*Trypanosoma, Plasmodium*), fungi (*Candida, Rhizopus, Blastomyces, Histoplasma, Cryptococcus*), viruses (influenza, ectromelia, encephalitis, and Newcastle disease), gram-positive bacteria (*Staphylococcus, Pneumococcus, Streptoccocus*), gram-negative bacteria (*Klebsiella, Pseudomonas, Salmonella, Escherichia coli*), and *Mycobacterium* has been demonstrated (4). Accordingly, it is reasonable to propose that endotoxin and endotoxin-like materials might alter the course of *N. fowleri* infections.

In this study, we have demonstrated that mice that survive primary infection with *N. flowleri* are highly resistant to intravenous (i.v.) challenge with a dose of organisms that produces uniformly fatal disease in normal control mice.

MATERIALS AND METHODS

Male DUB/ICR mice weighing 20 to 25 g were used in all experiments. Mice were obtained from Flow Research Animals, Inc., Dublin, Va. They

were allowed to adjust to their new environment for at least 1 week prior to experimentation. The mice were given free access to food (Purina Lab Chow, Purina Ralston Corp., St. Louis, Mo.) and water.

The protozoan culture *N. fowleri* (LEE strain) was kindly supplied by E. C. Nelson (Department of Microbiology, Virginia Commonwealth University). *N. fowleri* was grown in Nelson's medium consisting of Page amoeba saline (0.12 g of NaCl, 0.004 g of $MgSO_4 \cdot 7H_2O$, 0.004 g of $CaCl_2 \cdot 2H_2O$, 0.142 g of Na_2HPO_4, and 0.136 g of KH_2PO_4 per liter of distilled water) supplemented with 0.1% (wt/vol) liver infusion (Oxoid, London, England), 0.1% (wt/vol) glucose, and 2% (vol/vol) calf serum (Grand Island Biological Co., Grand Island, N.Y.). Medium (25 ml) in 75-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, Calif.) was inoculated and incubated for 96 h at 37 C.

The trophozoites were harvested by centrifugation at 4,000 × *g* for 10 min in a refrigerated centrifuge, washed with Page amoeba saline (described above), washed with 0.15 M NaCl, suspended in 0.15 M NaCl, and counted by using a hemocytometer. The cells were then diluted so that 0.2 ml would deliver the desired inoculum. All challenges with *N. fowleri* were given i.v. unless otherwise specified. Mice surviving *N. fowleri* given i.v. were divided into two groups. One-half of these mice were rechallenged i.v. with *N. fowleri* (5.0×10^6 trophozoites/mouse). Normal mice were also injected with *N. fowleri* intraperitoneally (i.p.) and subcutaneously. After 17 to 20 days, the mice challenged i.p. were rechallenged i.v. with 5.0×10^6 *Naegleria*/mouse.

All test materials were prepared so that 0.01 ml/g of mouse weight would provide the desired dose. All test materials were given by the i.p. route unless otherwise specified.

Escherichia coli O26:B6 lipopolysaccharide (LPS) (Boivin preparation of LPS; Difco Laboratories, Detroit, Mich.) was suspended in 0.15 M NaCl. Lipid A was extracted from *E. coli* 0127:B8 LPS by a procedure adapted from the method of Galanos et al. (8). The absence of 2-keto-3-deoxyoctonate was confirmed by the thiobarbiturate assay of Waravdekar and Saslaw (12) and was used to establish that hydrolysis was complete. Complexes of lipid A and bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) or concanavalin A (ConA) (grade IV; Sigma) were prepared by methods described by Galanos et al. (8). Aqueous lipid, solubilized with triethylamine, was complexed with an equal mass of BSA dissolved in water (2 mg of lipid + 2 mg of BSA per ml). The resulting mixture was dried in a rotary evaporator under reduced pressure. Complexes of BSA with myristic acid (Sigma) or *N,N*-dimethyl myristamide (Sigma) and complexes of ConA with β-hydroxymyristic acid (Applied Science Laboratories Inc., State College, Pa.) were also tested for endotoxic activity. Immediately before use the complexes were suspended in distilled water and dispersed with sonication.

RESULTS

The course of the disease after infection with *N. fowleri* is dependent upon dose, whether administered via the i.p. or i.v. route. After i.v. administration of 10^7 trophozoites/mouse, 100% of the mice were dead by day 10, as compared with 70% mortality on day 10 for a dose of 5.0×10^6 *Naegleria*/mouse and 40% mortality on day 10 for a dose of 2.5×10^6 trophozoites/mouse (Fig. 1). All of the mice in the group receiving 5.0×10^6 trophozoites/mouse were dead by day 13.

The usual signs of infection, such as ruffled fur and weight loss, were not observed in mice infected i.v. with *N. fowleri*. However, 1 or 2 days prior to death they displayed posterior paralysis, with no effect on the pectoral appendages. Occasionally acute deaths occurred within 1 or 2 h of *N. fowleri* administration. No signs of central nervous system involvement were seen with the acute deaths.

Mice injected with 5.0×10^6 and 10^7 trophozoites by the i.p. route had 10% mortality and no mortality by day 18, respectively (Fig. 2). However, all of the mice receiving 4.85×10^7 *N. fowleri* i.p. were dead by day 8. Acute deaths were observed in mice inoculated with 4.85×10^7 trophozoites/mouse with no signs of central nervous system involvement. On day 6, however, central nervous system involvement became apparent, similar to the effects seen in mice challenged i.v. with *N. fowleri*. Mice in-

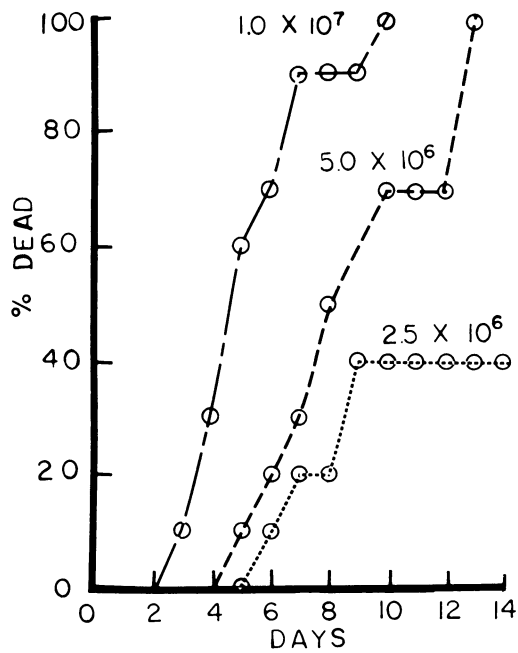

FIG. 1. *Time of death of mice given various i.v. doses of N. fowleri. There were 10 mice in each experimental group.*

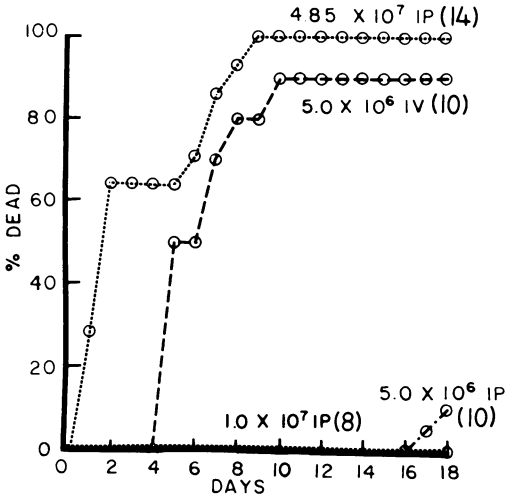

FIG. 2. *Time of death of mice given various i.p. doses of N. fowleri. Naegleria (5 × 10⁶/mouse) was administered i.v. as a control. The number of mice in each experimental group is given in parentheses.*

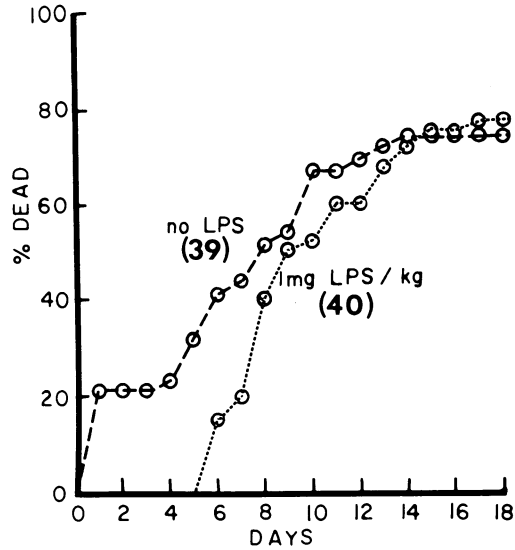

FIG. 3. *Effect of endotoxin on mortality of mice infected with Naegleria. Mice received 1 mg of E. coli O26:B6 LPS per kg i.p. 24 h prior to i.v. challenge with N. fowleri. Naegleria (5 × 10⁶/mouse) was administered i.v. to control mice. The number of mice in each experimental group is given in parentheses.*

jected with 5.0 × 10⁶ *N. fowleri* subcutaneously did not die or show signs of central nervous system involvement.

Endotoxin and complexes (using portions of the endotoxin molecule complexed to protein carriers) were surveyed to determine whether protection could be elicited in mice infected with *N. fowleri*. Endotoxin administered at a dose of 1 mg/kg 24 h prior to *N. fowleri* afforded some protection early in the infection (Fig. 3). By day 8 there was not any significant difference in survival.

Mice were pretreated with complexes of lipid A with BSA or ConA, myristic acid-BSA, *N,N*-dimethylmyristamide-BSA, or β-hydroxymyristic acid-ConA and challenged with *N. fowleri*. None of the above agents provided significant protection against naeglerial challenge (Table 1); for example, no significant protection was afforded by a complex of lipid A with ConA (Fig. 4).

Mice surviving a primary i.v. dose of *N. fowleri* were held for a period of 14 days with no death. The mice were divided into two groups, and one group was rechallenged i.v. with 5 × 10⁶ *N. fowleri*/mouse. Most normal control mice given a primary dose of *N. fowleri* died within a period of 5 to 14 days, as expected. The mice that survivied a primary dose of *N. fowleri* were significantly protected from the second challenge (Fig. 5); only one out of the seven mice in this group died. All but one of such survivors held as uninoculated controls lived.

Mice surviving a primary challenge i.p. with *N. fowleri* were rechallenged with 5 × 10⁶ trophozoites/mouse i.v. 20 days after the primary inoculation. Eight of such survivors held as uninoculated controls lived. Normal control mice receiving a primary dose of 5 × 10⁶ *N. fowleri* i.v. died between days 7 and 14. Only 1 of 10 mice that survived a primary i.p. injection of *N. fowleri* failed to survive i.v. rechallenge with *N. fowleri*; it died on day 11 post-i.v. inoculation. Thus, significant protection was afforded mice pretreated i.p. with *N. fowleri* and then rechallenged i.v. (Fig. 6).

TABLE 1. *Test substances that did not alter the course of fatal disease after infection with N. fowleri*[a]

Test substance	No. of mice tested
BSA	31
BSA-*N,N*-dimethylmyristamide	20
BSA-lipid A	27
BSA-myristic acid	20
ConA	20
ConA-β-hydroxymyristic acid	30
ConA-lipid A	20
N,N-dimethylmyristamide	20
β-Hydroxymyristic acid	17
Myristic acid	20

[a] Adult male DUB/ICR mice were given 50 mg of the test material per kg i.p. 24 h prior to an i.v. challenge with 5 × 10⁶ *N. fowleri* trophozoites/mouse.

DISCUSSION

The intranasal route is commonly used for inoculating mice or most experimental animals with *N. fowleri*, since it has been thought to be the route of entry in primary amoebic meningoencephalitis in humans (5). However, with intranasal inoculation it is difficult to quantitate the number of trophozoites actually retained in the host. Culbertson et al. (6) inoculated mice with *N. fowleri* by the i.p., i.v., and intracerebral routes as well as intranasally. They stated that wide hematogenous dissemination was seen after i.v. and i.p. administration, but no data were presented (6). Carter (2) also studied the pathogenicity of *N. fowleri* administered by various routes. Swiss-Webster mice were inoculated by the intranasal, intragastric, intrarectal, i.v., intramuscular, subcutaneous, i.p., intrahepatic, intrapleural, anterior intracerebral, and posterior intracerebral routes. A dose of 2×10^4 *N. fowleri* in proteose peptone-glucose medium with a pH of 6.5 and a molarity of 0.135 M was administered to each of three mice per group. Clinical symptoms and death occurred only in mice infected with *N. fowleri* by the intranasal, anterior intracerebral, or posterior intracerebral routes. One of three mice died with no clinical symptoms in both of the groups given *N. fowleri* i.v. and intrahepatically. No clinical or pathological evidence of disease was found when *N. fowleri* was administered via the intrarectal, intragastric, intramuscular, i.p., subcutaneous, or intrapleural routes (2).

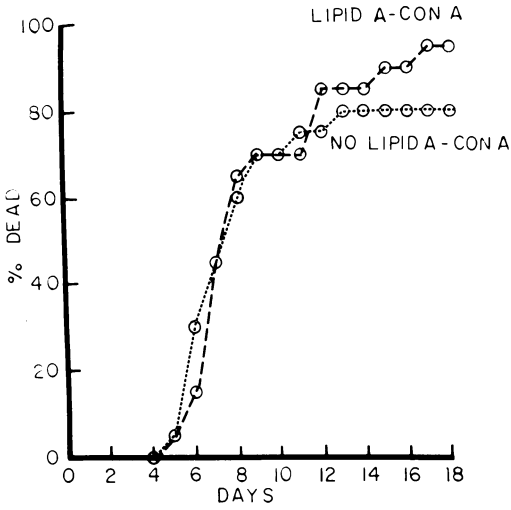

FIG. 4. *Effect of lipid A-ConA on mortality of mice infected with Naegleria. Mice received 50 mg of lipid A-ConA per kg i.p. 24 h prior to i.v. challenge with N. fowleri. Naegleria (5 × 10⁶/mouse) was administered i.v. to control mice. There were 20 mice in each experimental group.*

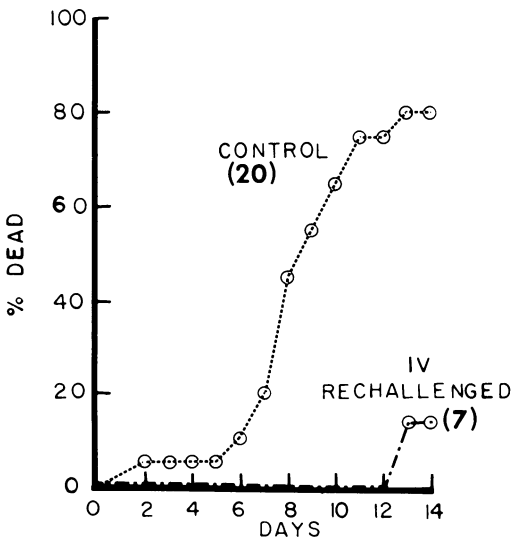

FIG. 5. *Immunity of mice that survived primary i.v. infection with Naegleria. Mice administered N. fowleri i.v. were rechallenged after 2 weeks with 5 × 10⁶ Naegleria/mouse i.v. The number of mice in each experimental group is given in parentheses.*

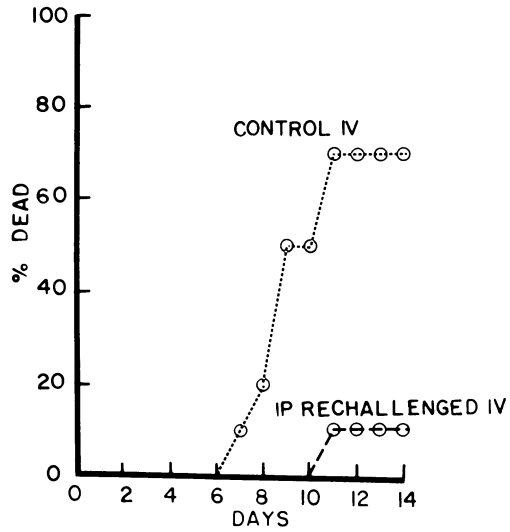

FIG. 6. *Immunity of mice after intraperitoneal infection with Naegleria. Mice given N. fowleri i.p. were rechallenged after 2 weeks with 5 × 10⁶ Naegleria/mouse i.v. There were 10 mice in each experimental group.*

The literature on route of inoculation of *N. fowleri* is not convincing because too few animals were included in the experimental group, the numbers of trophozoites administered were not reported or too few amoebae were administered, and the age of the *N. fowleri* culture was not stated. In our study mice were inoculated by the subcutaneous, i.p., and i.v. routes. Clinical symptoms and death were produced in mice inoculated via the i.v. and i.p. routes. Only one dose (5×10^6 trophozoites/mouse) was administered subcutaneously. Since the outcome of an infection with *N. fowleri* via the i.v. and i.p. routes is dose dependent, it is reasonable to propose that a larger dose can produce the disease in mice via subcutaneous inoculation.

Mice pretreated with bacterial LPS 24 h prior to i.v. challenge with *N. fowleri* were afforded protection early in the course of the disease. Mice pretreated with LPS were definitely protected from acute death, which was occasionally observed in mice inoculated i.v. with *N. fowleri* alone. Acute death may have been caused by the toxin that is reportedly produced by *N. fowleri* (3). None of the other endotoxin-like substances tested afforded protection from *N. fowleri* infection in mice.

According to Lüderitz et al. (9), lipid A is the active component of LPS. They reported that lipid A complexed with certain carriers is less active than the parent LPS but shows endotoxic activity in the following assays: mouse lethality, pyrogenicity, bone marrow necrosis, *Limulus* gelation, and complement inactivation (9). Thus it was disappointing that the lipid A complexes showed no alteration of the course of the naeglerial infection.

Mice that survived a primary dose of *Naegleria* i.v. or i.p. were significantly protected from a rechallenge of *Naegleria* i.v. This effect can probably be attributed to increased immunity of the host to naeglerial infection.

ACKNOWLEDGMENT

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